RESPONSE

I. Status of the Claims

Claims 3-9 are presently pending in this case.

II. Rejection of Claims 3-9 Under 35 U.S.C. § 101

The Action rejects claims 3-9 under 35 U.S.C. § 101, allegedly because the claimed invention lacks support by either a specific and substantial asserted utility or a well established utility. Applicants respectfully traverse.

The Action discounts many of the numerous utilities described in the specification for the claimed sequences of the present invention based on the position that while credible, these utilities are not specific or substantial. While Applicants in no way agree with the Examiner's arguments, Applicants have chosen to expand on only a few of the utilities presented as only one is required and incorporate by reference those made in earlier responses.

Applicants respectfully submit that the legal test for utility involves an assessment of whether those skilled in the art would find any of the utilities described for the invention to be credible or believable. According to the Examination Guidelines for the Utility Requirement, if the applicant has asserted that the claimed invention is useful for any particular purpose (i.e., it has a "specific and substantial utility") and the assertion would be considered credible by a person of ordinary skill in the art, the Examiner should not impose a rejection based on lack of utility (66 Federal Register 1098, January 5, 2001).

In *In re Brana,* (34 USPQ2d 1436 (Fed. Cir. 1995), "*Brana*"), the Federal Circuit admonished the P.T.O. for confusing "the requirements under the law for obtaining a patent with the requirements for obtaining government approval to market a particular drug for human consumption". *Brana* at 1442. The Federal Circuit went on to state:

> At issue in this case is an important question of the legal constraints on patent office examination practice and policy. The question is, with regard to pharmaceutical inventions, what must the applicant provide regarding the practical utility or usefulness of the invention for which patent protection is sought. This is not a new issue; it is one which we would have thought had been settled by case law years ago.

Brana at 1439, emphasis added. The choice of the phrase "utility or usefulness" in the foregoing quotation is highly pertinent. The Federal Circuit is evidently using "utility" to refer to rejections under 35 U.S.C. § 101, and is using "usefulness" to refer to rejections under 35 U.S.C. § 112, first paragraph. This is made evident in the continuing text in *Brana*, which explains the correlation between 35 U.S.C. §§ 101 and 112, first paragraph. The Federal Circuit concluded:

> FDA approval, however, is not a prerequisite for finding a compound useful within the meaning of the patent laws. Usefulness in patent law, and in particular in the context of pharmaceutical inventions, necessarily includes the expectation of further research and development. The stage at which an invention in this field becomes useful is well before it is ready to be administered to humans. Were we to require Phase II testing in order to prove utility, the associated costs would prevent many companies from obtaining patent protection on promising new inventions, thereby eliminating an incentive to pursue, through research and development, potential cures in many crucial areas such as the treatment of cancer.

Brana at 1442-1443, citations omitted. In assessing the question of whether undue experimentation would be required in order to practice the claimed invention, the key term is "undue", not "experimentation". *In re Angstadt and Griffin*, 190 USPQ 214 (C.C.P.A. 1976). The need for some experimentation does not render the claimed invention unpatentable. Indeed, a considerable amount of experimentation may be permissible if such experimentation is routinely practiced in the art. *In re Angstadt and Griffin, supra*; *Amgen, Inc. v. Chugai Pharmaceutical Co., Ltd.*, 18 USPQ2d 1016 (Fed. Cir. 1991). As a matter of law, it is well settled that a patent need not disclose what is well known in the art. *In re Wands*, 8 USPQ 2d 1400 (Fed. Cir. 1988).

Even under the newly installed utility guidelines, Applicants note that MPEP 2107 (II)(B)(1) states:

> (1) If the applicant has asserted that the claimed invention is useful for any particular practical purpose (i.e., it has a "specific and substantial utility") and the assertion would be considered credible by a person of ordinary skill in the art, do not impose a rejection based on lack of utility. (MPEP 2107 (II)(B)(1))

Based on the statement "The claimed invention in the instant case is drawn to nucleic acid sequences, not a device" (Action page 14, lines 20-21) , the Examiner is apparently of the opinion that the various mandatory legal precedents provided in Federal Circuit decisions are narrowly limited to the particular technologies discussed in each specific case. If this were indeed true, which is clearly not the case, the Federal Circuit's various articulations of the legal standards for utility, enablement, doctrine of equivalents, etc., for juice dispenser inventions, for example, would have virtually no bearing on similar legal inquiries relating to electrical inventions, business methods, or inventions from distinct arts. Applicants again contend that the Examiner's articulated position lacks any legal and procedural foundation and is intellectually unsound. Indeed, one might presume to speculate that a Federal Circuit panel would find the Examiner's stated position as representing a rather remarkable deviation from established legal precedent.

In the present Action, the Examiner states that "the skilled artisan would not be able to categorize the polynucleotide and polypeptide of the instant application as a G-protein coupled receptor"(Action at page 4, lines 2-4). This is clearly incorrect, as Applicants have previously presented evidence the claimed sequences have been identified by third party scientists *wholly unaffiliated with Applicants* as human olfactory receptor 2B6 (Hs6M1-32) (Olfactory receptor 6-31) (alignment and report provided previously) and has been the subject of several later patent applications which also identify the claimed sequences as those encoding human G protein-coupled receptors (WO200166746 and WO200127158). Thus clearly, as those of skill in the art when faced with the same sequences as those claimed in the present application readily identified then as those encoding a G protein-coupled receptor, then clearly those of skill in the art, in addition to Applicants, were in fact "able to categorize the polynucleotide and polypeptide of the instant application as a G-protein coupled receptor" as was demonstrated by the evidence provided in a previous response. Clearly, Applicants assertions were credible as they have been corroborated by an unaffiliated third party.

As taught in the application and as well known to those of skill in the art, G protein coupled receptors (GPCRs) play a critical role in, *intra alia*, signal transduction and cell activation. In fact, many oncogenes are linked to GPCRs. In addtion, GPCRs are, as evidenced by previously submitted exhibits GPCRs are prominent drug targets with approximately 60% of marketed prescription drugs being directed at them. The recognition of odorants by G protein coupled

olfactory receptors is the first stage in odor discrimination (Krautwurst, *et al.*, Cell 95(7):917-26, **Exhibit A**). Agonists and antagonists directed at this novel human G protein coupled receptor would be expected to effect feeding behavior and thus potentially address, *inter alia,* obesity, anorexia or cachexia and other feeding disorders.

The Action again cites (at page 3, lines 10 - 22) the article by Ji *et al.* ("Ji"; 1998, J. Biol. Chem. *273*:17299-17302), as teaching that structural homology alone is not a good predictor of function and that different human G protein coupled receptor families show diverse modes of activity. As noted in a previous response these quotes again suggest that activities of members of a G-protein coupled receptors within a subfamily are similar and the fact that there is little or no homology between subfamilies is irrelevant. Therefore, Ji does not argue against the utility of the presently claimed sequences. Applicants therefore submit that given the state of understanding that exists for GPCRs, that the sequences of the present invention clearly have specific and substantial utility and thus meet the requirements of 35U.S.C. § 101.

Furthermore, the Examiner also objects to the lack of allegedly required evidence regarding description "the specification of the instant application does not teach the skilled artisan which domains of the NGPCR polynucleotide and polypeptide are structurally characteristic of G protein-coupled receptors" (Action at page 4, lines 4-6) .

First, Applicants note that it has long been established that "there is no statutory requirement for the disclosure of a specific example". *In re Gay*, 135 USPQ 311 (C.C.P.A. 1962). The Action goes on to suggest that the invention lacks utility because the disclosure provides no guidance as to where the important structural elements of the protein encoded by the sequences of the present invention that are essential to its function are located. This concern is also misplaced, as it is well established that "an inventor is not required to understand the theory of how his invention works". *Micro Motion, Inc. v. Exac Corp.*, 16 USPQ2d 1001, 1013 (Cal. 1990).

Second, Applicants note that whether or not the specification as filed taught to the Examiner's satisfaction "which domains of the NGPCR polynucleotide and polypeptide are structurally characteristic of G protein-coupled receptors" those domains are present and are an inherent property of the claimed sequences as the domains are encoded by the claimed sequences. Similarly as the functional domains are inherent to the claimed sequences which encode them, thus so is the function of a protein inherent to the sequences which encode that protein. Thus there is no

need to identify the domains that are present in the claimed sequences, because these domains are inherent features of the claimed sequences.

The Action also reiterates the position that the use of the presently claimed polynucleotides, as in DNA chips and gene mapping, is not specific or substantial. Further, the Action seems to be requiring Applicants to identify the biological role of the nucleic acid or function of the protein encoded by the presently claimed polynucleotides before the present sequences can be used in gene chip applications that meet the requirements of § 101. Applicants respectfully point out that knowledge of the exact function or role of the presently claimed sequence **is not required** to track expression patterns using a DNA chip. As set forth in Applicants First Response, given the widespread utility of such "gene chip" methods using *public domain* gene sequence information, there can be little doubt that the use of the presently described *novel* sequences would have great utility in such DNA chip applications. The claimed sequence provides a specific marker of the human genome (see evidence below), and that such specific markers are targets for discovering drugs that are associated with human disease. Thus, those skilled in the art would instantly recognize that the present nucleotide sequence would be an ideal, novel candidate for assessing gene expression using, for example, DNA chips, as the specification details at least on page 9. Such "DNA chips" clearly have utility, as evidenced by hundreds of issued U.S. Patents, as exemplified by U.S. Patent Nos. 5,445,934, 5,556,752, 5,744,305, as well as more recently issued U.S. Patent Nos. 5,837,832, 6,156,501 and 6,261,776. Accordingly, the present sequence has a specific utility in such DNA chip applications. Clearly, compositions that enhance the utility of such DNA chips, such as the presently claimed nucleotide sequence, must also be useful.

Additionally, since only a small percentage of the genome (2-4%) actually encodes exons, which in-turn encode amino acid sequences. Thus, not all human genomic DNA sequences are useful in such gene chip applications, further discounting the Examiner's position that such uses are "generic". Thus, the present claims clearly meet the requirements of 35 U.S.C. § 101. It has been clearly established that a statement of utility in a specification must be accepted absent reasons why one skilled in the art would have reason to doubt the objective truth of such statement. *In re Langer*, 503 F.2d 1380, 1391, 183 USPQ 288, 297 (CCPA, 1974); *In re Marzocchi*, 439 F.2d 220, 224, 169 USPQ 367, 370 (CCPA, 1971).

Evidence of the "real world" substantial utility of the present invention is further provided by the fact that there is an entire industry established based on the use of gene sequences or fragments thereof in a gene chip format. Perhaps the most notable gene chip company is Affymetrix. However, there are many companies which have, at one time or another, concentrated on the use of gene sequences or fragments, in gene chip and non-gene chip formats, for example: Gene Logic, ABI-Perkin-Elmer, HySeq and Incyte. In addition, one such company, Rosetta Inpharmatics, was viewed to have such "real world" value that it was acquired by large pharmaceutical company, Merck & Co., for substantial sums of money (net equity value of the transaction was $620 million). The "real world" substantial industrial utility of gene sequences or fragments would, therefore, appear to be widespread and well established. Clearly, persons of skill in the art, as well as venture capitalists and investors, readily recognize the utility, both scientific and commercial, of genomic data in general, and specifically human genomic data. Billions of dollars have been invested in the human genome project, resulting in useful genomic data (see, *e.g.*, Venter *et al.*, 2001, Science *291*:1304). The results have been a stunning success as the utility of human genomic data has been widely recognized as a great gift to humanity (see, *e.g.*, Jasny and Kennedy, 2001, Science *291*:1153). Clearly, the usefulness of human genomic data, such as the presently claimed nucleic acid molecules, is substantial and credible (worthy of billions of dollars and the creation of numerous companies focused on such information) and well-established (the utility of human genomic information has been clearly understood for many years).

Further evidence previously submitted by Applicants of utility of the presently claimed polynucleotide is the utility the present nucleotide sequence has a specific utility in determining the genomic structure of the corresponding human chromosome, for example mapping the protein encoding regions, as described in the specification at least at page 14, line 1 and previously evidenced. Clearly, the present polynucleotide provides exquisite specificity in localizing the specific region of the human chromosome containing the gene encoding the given polynucleotide, a utility not shared by virtually any other nucleic acid sequences. In fact, it is this specificity that makes this particular sequence so useful. Early gene mapping techniques relied on methods such as Giemsa staining to identify regions of chromosomes. However, such techniques produced genetic maps with a resolution of only 5 to 10 megabases, far too low to be of much help in identifying specific

genes involved in disease. The skilled artisan readily appreciates the significant benefit afforded by markers that map a specific locus of the human genome, such as the present nucleic acid sequence.

Only a minor percentage of the genome actually encodes exons, which in-turn encode amino acid sequences. The presently claimed polynucleotide sequence provides biologically validated empirical data (*e.g.*, showing which sequences are transcribed, spliced, and polyadenylated) that *specifically* define that portion of the corresponding genomic locus that actually encodes exon sequence. Equally significant is that the claimed polynucleotide sequence defines how the encoded exons are actually spliced together to produce an active transcript (*i.e.*, the described sequences are useful for functionally defining exon splice-junctions). The Applicants respectfully submit that the practical scientific value of expressed, spliced, and polyadenylated mRNA sequences is readily apparent to those skilled in the relevant biological and biochemical arts. For further evidence in support of the Applicants' position, the Board is requested to review, for example, section 3 of Venter *et al.* (*supra at* pp. 1317-1321, including Fig. 11 at pp.1324-1325), which demonstrates the significance of expressed sequence information in the structural analysis of genomic data. The presently claimed polynucleotide sequence defines a biologically validated sequence that provides a unique and specific resource for mapping the genome essentially as described in the Venter *et al.* article.

The Examiner questions these asserted utilities, assuming the position that the use of expression profiling or assessing gene expression patterns is not available in this specification in a 'practical' or 'real world' manner because the expression of the protein has not been associated with any drug treatment/ disease diagnosis. Applicants respectfully point out that in many instances nucleotides are included on a gene chip exactly because the sequence "has not been associated with any drug treatment/ disease diagnosis". In fact, numerous companies throughout the biotechnology industry have paid millions of dollars to companies such as Affymetrix for gene chips that include nucleotide sequences that have "not been associated with any drug treatment/ disease diagnosis". Therefore, the Examiner's arguments are particularly difficult to comprehend, unless it is the position of the Office that companies such as Affymetrix do not exist in the "real world". Applicants therefore submit that given the actual "practical" and "real world" utility of expression profiling using gene chips, the present invention clearly meets the requirements of 35U.S.C. § 101.

The Examiner further states, with regard to the utility of the claimed sequences in genomic mapping that while the "Applicant indicates that the sequences in the instant specification (SEQ ID NOs:1 and 2) are specific marker of the human genome, the specification does not teach if the entire sequences are to be used as markers or sections thereof" (page 10, lines 16-18). This is again incorrect. The claims read on full-length sequences, such properties are inherent in the claimed sequences, which were previously demonstrated to have utility in localizing the specific region of the human chromosome 6 and identification of functionally active intron/exon splice junctions by the evidence previously submitted in which the specific utility of the sequences of the present invention was demonstrated with a blast analysis using the SEQ ID NO:1 of the present invention indicates that the coding the sequence is present on chromosome 6 and contained within clone AL133267. Thus clearly the claimed sequences have specific and substantial utility in mapping the human genome.

Still further, the Action also disregards (on page 12) that the present situation is akin to that regarding the patenting of tires and golf balls. Applicants respectfully submit that in both situations, while the inventions are capable of being used for a similar purpose, each also has properties unique to it. For example the present sequences encode a novel human G protein-coupled receptor with the expression pattern described in the application. While such a sequence is indeed like others in that it is a sequence (as new golf balls are like previous golf balls, both are golf balls and can be used in the game of golf) it is also unique, in that there are features of the claimed sequences that are not shared by other sequences (as is the case with the 'high flying' golf ball). Therefore Applicants note that like golf balls and tires the claimed sequences meet the requirements of 35U.S.C. § 101.

The Action next cites *Brenner v Manson*, 383 U.S. 519, 148 USPQ 689 (Sup. Ct. 1966), for noting that "a patent is not a hunting license. It is not a reward for the search, but compensation for its successful conclusion." Applicants whole heartedly agree and note that the search for the sequences that are the claimed invention of the present application is over. Applicants were the first to have successfully concluded the search for the presently claimed novel human G protein-coupled receptor sequences and the results of this search have been clearly listed in the Sequence Listing of the instant application. No further "hunting" is required. As Applicants have brought this search to its successful conclusion, they are due the reward, a U.S. Patent claiming the results of their difficult

but successful efforts. Thus, Applicants respectfully request the withdrawal of the rejection of the claimed sequences under 35U.S.C. § 101.

Finally, while Applicants acknowledge the Examiner's position that she has no authority to comment on the legality of the Guidelines and the validity of U.S. Patents. However, Applicants again note that it is an accepted tenant of U.S. law that issued U.S. patents carry with them the presumption of a legal presumption of validity which in this case indicates that the inventions claimed in the cited patents are *legally presumed* to be in full compliance with the provisions of 35 U.S.C. sections 101, 102, 103, and 112. Applicants understanding is that issued United States patents retain a legal presumption of validity which in this case indicates that the inventions claimed in the cited patents are *legally presumed* to be in full compliance with the provisions of 35 U.S.C. sections 101, 102, 103, and 112. Applicants are aware that the USPTO alleges that New Utility Guidelines were derived from prior practice, yet clearly, in the present case at least they result in disparate treatment to the point that they suggest a change in the law. Applicants respectfully submit that, absent a change in the law as enacted by Congress and signed by the President, it is improper for the Examiner to hold Applicants' invention to a different legal standard of patentability. Given the rapid pace of development in the biotechnology arts, it is difficult for Applicants to understand how an invention fully disclosed and free of prior art at the time the present application was filed, could somehow retain *less* utility and be *less* enabled than inventions in the cited issued U.S. patents (which were filed during a time when the level of skill in the art was clearly lower). Simply put, Applicants invention is *more* enabled and retains *at least as much* utility as the inventions described in the claims of the U.S. patents of record. Any argument to the contrary is at best arbitrary and at worst capricious. Absent authority provided by an act of Congress or Executive order, arbitrary or capricious conduct by an administrative office the U.S. government has historically proven to conflict with the provisions of the U.S. Constitution. The Patent Office does not have the authority to rewrite U.S. law. However, the Patent Office does have a Constitutional obligation to administer U.S. law in an unbiased and procedurally consistent manner. That is what the Applicants are respectfully requesting the Examiner to consider in the present matter. With regard to the issue of the legality of the New Utility Guidelines, Applicants assume this will eventually be resolved by the Courts, as has been the case with past USPTO rule determinations.

Further it should be noted that it appears that one of the intents of the New Utility Guidelines appears to be to align U.S. patent law with that of the rest of the world. But clearly the new Guidelines fail in this regard, for the European Patent Office has determined that the identification of novel human G protein-coupled receptor sequences, in view of the prior art, is obvious and therefore requires no inventive skill. If an invention is deemed obvious and requires no inventive step, then clearly it must satisfy the requirements under 35U.S.C. § 101.

For each of the foregoing reasons, Applicants submit that, in light of the above discussion and those presented previously, the presently claimed invention has been shown to have a substantial, specific, credible and well-established utility and that the rejection of pending claims 3-9 under 35 U.S.C. § 101 has been avoided, and respectfully request that the rejection be withdrawn.

III. Conclusion

The present document is a full and complete response to the Final Office Action. In conclusion, Applicants submit that, in light of the foregoing remarks, the present case is in condition for allowance, and such favorable action is respectfully requested. Should Examiner Bunner have any questions or comments, or believe that certain amendments of the claims might serve to improve their clarity, a telephone call to the undersigned Applicants' representative is earnestly solicited.

Respectfully submitted,

August 16, 2004
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EXHIBIT A

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Identification of Ligands for Olfactory Receptors by Functional Expression of a Receptor Library

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Summary

The recognition of odorants by olfactory receptors represents the first stage in odor discrimination. Here, we report the generation of an expression library containing a large and diverse repertoire of mouse olfactory receptor sequences in the transmembrane II–VII region. From this library, 80 chimeric receptors were tested against 26 odorants after transfection into HEK-293 cells. Three receptors were identified to respond to micromolecular concentrations of carvone, (−) citronellal, and limonene, respectively. We also found that the mouse I7 receptor, unlike the rat I7 receptor, prefers heptanal instead of octanal, as a result of a single valine-to-isoleucine substitution. This finding represents the beginning of a molecular understanding of odorant recognition. The identification, on a large scale, of cognate receptor–odorant interactions should provide insight into olfactory coding mechanisms.

Introduction

Olfactory receptors located on the specialized cilia of olfactory neurons bind odorants and initiate the transduction of chemical stimuli into electrical signals. This olfactory transduction process is now quite well understood. It involves a G protein–coupled activation of an adenylyl cyclase, which leads to a rise in cAMP and consequently the opening of cyclic nucleotide–activated, nonselective cation channels (see for example, Reed, 1992). These open channels produce a cation influx that results in the depolarization of the olfactory neuron (Nakamura and Gold, 1987; Firestein and Werblin, 1989; Kurahashi, 1989). The identifications of specialized isoforms of Gα, adenylyl cyclase, and the cyclic nucleotide–activated channel in the olfactory cilia have strongly supported the importance of this pathway (Jones and Reed, 1989; Bakalyar and Reed, 1990; Dhallan et al., 1990; Ludwig et al., 1990). A second olfactory transduction mechanism has also been proposed, involving the generation of IP_3 and the opening of IP_3-activated channels on the ciliary plasma membrane (Boekhoff et al., 1990; Schild et al., 1995). However, recent experiments involving genetically modified mice have indicated that the electroolfactogram, which reflects the mass response of sensory neurons in the olfactory epithelium, is generated by the cAMP pathway (Brunet et al., 1996; Belluscio et al., 1998).

The mechanism underlying our ability to detect and discriminate thousands of odorants became clearer after it was found that the putative olfactory receptors were 7-transmembrane-domain (TMD) receptors belonging to a multigene family with perhaps 500–1000 members (Buck and Axel, 1991; Levy et al., 1991; Ben-Arie et al., 1994; Sullivan et al., 1996). Furthermore, studies with in situ hybridization have suggested that each olfactory receptor neuron may express only one, or at most a few, of these olfactory receptor proteins (Ressler et al., 1993; Vassar et al., 1993). Thus, odorant discrimination for a given receptor neuron should depend on the ligand specificity of the one or few receptor proteins it expresses. At the same time, electrophysiological studies on single, dissociated olfactory neurons have suggested that individual cells can detect a broad spectrum of odorants, in that any given cell has a high probability of responding to a small, arbitrary set of odorants (Firestein et al., 1993). Taken together, these results suggest that each olfactory receptor protein responds to a large number of odorants.

In order to address the question of odorant–receptor interaction properly, it is necessary to identify the odorants that a particular olfactory receptor recognizes. Despite the many putative olfactory receptors that have been cloned, only limited progress has been made in the functional expression of these receptors. Expression of the rat OR5 receptor in insect cells results in a modest elevation on second messengers when exposed to a mixture of odorants (Raming et al., 1993). However, responses to single compounds have not been reported in this system. In an alternative approach, the rat I7 receptor has been introduced into an adenovirus expression vector and used to infect rat olfactory neurons in vivo (Zhao et al., 1998). In this study, the infected animals were found to give an electroolfactogram significantly larger than that of control animals when stimulated by the odorant octanal; structurally related compounds produced a similar but weaker effect. Finally, the *C. elegans* ODR-10 receptor, identified by behavioral studies to respond specifically to diacetyl (Sengupta et al., 1996), has been expressed in HEK-293 cells and found to recognize the same odorant in a Ca^{2+}-imaging assay (Wellerdieck et al., 1997; Zhang et al., 1997). Despite this progress, information about odorant–receptor recognition remains meager, especially considering that there are hundreds of olfactory receptors and thousands of odorants.

The expression of cloned olfactory receptors in heterologous systems to identify their corresponding ligands has been complicated by the failure of these proteins to translocate efficiently to the plasma membrane. Studies on *C. elegans* and *Drosophila* have suggested the importance of accessory proteins in the proper localization of 7-TMD receptors to the sensory structures of receptor neurons (Colley et al., 1991; Stamnes et al., 1991; Dwyer et al., 1998). An attempt has been made

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to identify critical regions required for the targeting of olfactory receptors to the plasma membrane by generating chimeras of these receptors with the β_2-adrenergic receptor (McClintock et al., 1997).

We have devised an approach that allows us to generate rapidly a large number of olfactory receptor sequences suitable for functional expression in HEK-293 cells. This expression cloning approach stems from knowledge of the mechanism of ligand binding in two model systems. In the case of the β_2-adrenergic receptor, critical residues for agonist binding and receptor activation have been identified in TM II, III, V, and VI. Cross-linking studies have further identified particular residues within these regions that form the binding pocket and directly contact the ligand (Strader et al., 1994). For the visual pigments, the 11-*cis* retinal moiety, which controls activation of the pigment, is covalently linked through a Schiff base to a lysine residue in TM VII of the opsin (Nathans and Hogness, 1984). Additional residues located in TM IV and VI interact with the chromophore to determine the absorption spectrum for a given pigment (Merbs and Nathans, 1993). Studies on additional G protein–coupled receptors that recognize small ligands also support the notion that ligand binding is largely restricted to the transmembrane regions (Strader et al., 1994). Moreover, the high degree of diversity in these regions of olfactory receptors, as noted by Buck and Axel (1991), is consonant with the idea that these regions contribute to the recognition of a broad repertoire of ligands by the receptors. Accordingly, we employed a PCR strategy that allowed us to clone efficiently the TM II–VII region of a significant fraction of the mouse olfactory receptor family in an expression vector and use them for functional studies.

In order to facilitate functional expression in HEK-293 cells, we took advantage of the finding that rhodopsin shows a high level of expression in this system as well as efficient translocation to the plasma membrane (Sung et al., 1991; see also Figure 2A, leftmost panel). The expression constructs we generated all had a rhodopsin N-terminal extension that we found empirically to facilitate translocation of the synthesized proteins to the plasma membrane.

Our approach provides a model system for the study of ligand specificity and structure–function relationships for olfactory receptors.

Results

Cloning of TM II–VII Chimeric Receptors

In initial experiments, the 5′-untranslated region of the rhodopsin gene, together with its coding region for the initiation methionine and the next 19 residues, were joined to a full-length cDNA for the olfactory receptor M4 (Qasba and Reed, 1998) and expressed in HEK-293 cells under the control of the CMV promoter. Based on immunostaining with the B6-30 monoclonal antibody directed against the N-terminal 15 residues of rhodopsin (Hargrave et al., 1986), a fraction of the chimeric protein appeared to be localized on the plasma membrane (data not shown). These results prompted us to exploit this property by including the rhodopsin N terminus in the chimeric receptors in our expression cloning of olfactory receptors.

A PCR-based amplification strategy taking advantage of the homology shared among olfactory receptors at the beginning of TM II and the end of TM VII was used to generate a product containing a large number of olfactory receptor sequences. Because previous studies have localized critical residues for ligand binding in other G protein–coupled receptors between TM II and TM VII (see Introduction), we reasoned that the insertion of a cloned TM II–VII segment into an expression vector backbone might impart to the resulting chimeric protein the ligand specificity of the corresponding full-length receptor. The structure of the overall construct, pCMV-Rho/M4$_{NC}$, is shown in Figure 1A. The degenerate oligonucleotides are flanked by the coding sequences for the appropriate regions of the mouse M4 receptor. The PCR product amplified from mouse olfactory cDNA was inserted between the PstI and BspEI sites, and the resulting clones were arrayed into 480 microtiter wells.

Sequence analysis of 26 random clones revealed that all but 3 were distinct sequences. Although each insert shared hallmarks of previously characterized olfactory receptors, the sequenced receptors were all new members of the olfactory receptor family and were distributed broadly (shown in boldface in Figure 1B) across a similarity dendrogram, which also depicts ten previously cloned olfactory receptors (Buck and Axel, 1991) (shown in italics in Figure 1B). Thus, the arrayed receptor plasmids represented a diverse library of olfactory receptor sequences amenable to expression studies.

Establishment of the Functional Expression System—Analysis of β_2-Adrenergic and Rat I7 Receptors

The functional analysis of expressed receptors requires a robust and sensitive assay system suitable for efficient screening with a large number of ligands. Although the established role of cAMP in olfactory signaling offers a biochemical approach involving measurement of cAMP production in response to odorant stimulation, an alternative, rapid assay would be to coexpress the cloned receptors with G$\alpha_{15,16}$ subunits, which can promiscuously couple 7-TMD receptors that normally signal through other second messengers to the PIP_2 pathway (Offermanns and Simon, 1995). In this reporter system, receptor activation leads to the generation of an IP_3-mediated increase in intracellular Ca^{2+}, which can be measured at the single-cell level with high sensitivity and good temporal resolution using the dye FURA-2 and ratiofluorometric imaging. These attributes were able to compensate for the low transfection efficiency in transient expression systems that would hinder more traditional biochemical assays.

As a test example, a construct with the TM II–VII region from the β_2-adrenergic receptor inserted in the pCMV-Rho/M4$_{NC}$ vector (Rho/M4$_{NC}$-β_2 TM II–VII) was cotransfected with G$\alpha_{15,16}$ into HEK-293 cells. Immunocytochemical localization with the B6-30 antibody against the rhodopsin tag indicated that at least a portion of the expressed protein appeared to be localized to the plasma membrane (Figure 2A, second panel from left).

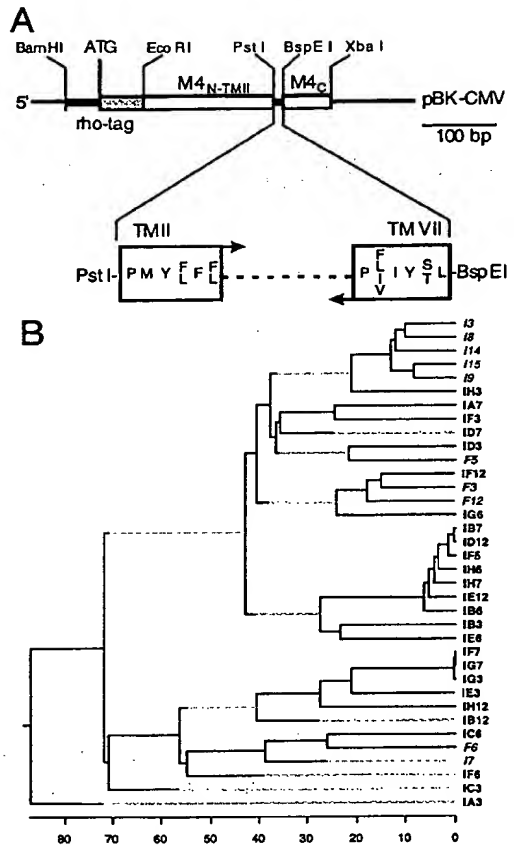

Figure 1. Generation and Analysis of a Library of Olfactory Receptor TM II–VII Fragments

(A) The mammalian expression construct, based on the modified pBK-CMV vector, contains a 5′-untranslated sequence and the coding region for the N-terminal 20 amino acids of rhodopsin (rho tag), followed by the sequence from the N terminus to the beginning of TM II of the mouse olfactory receptor M4 ($M4_{N\text{-}TM\,II}$) and the C-terminal sequence of the same receptor ($M4_C$). The PCR product coding for TM II–VII of a large number of olfactory receptors was obtained with degenerate oligonucleotide primers dk71 and dk72 on mouse olfactory epithelial cDNA. The 0.7 kb DNA product was cloned between the PstI and BspEI sites of the expression vector.

(B) Phylogenetic tree of 26 cloned mouse olfactory receptor TM II–VII sequences. Deduced amino acid sequences (boldface; 32%–99.5% identity with each other) were aligned by the ClustalW algorithm. For comparison, ten TM II–VII regions (in italics) from published rat olfactory receptors (Buck and Axel, 1991) were included with the mouse sequences in the alignment. Scale gives percentage of amino acid divergence as depicted by the black lines in the dendrogram.

Bath application of the adrenergic agonist isoproterenol to the transfected cells resulted in a transient increase in intracellular Ca^{2+} (Figure 2B). A second application of isoproterenol frequently failed to elicit a response (data not shown), possibly suggesting a rapid desensitization of the $G\alpha_{15,16}$-mediated signal transduction pathway. Although its mechanism is unclear, this rapid desensitization was a frequent observation with this expression system (see below and Zhang et al., 1997). HEK-293 cells have intrinsic muscarinic receptors coupled to the PIP_2 pathway via endogenous G proteins (Offermanns et al., 1994). The rise in intracellular Ca^{2+} upon activation of this pathway by bath-applied acetylcholine (10 μM; see Figures 2B–2E) serves as a control in this system. The Ca^{2+} transient induced by isoproterenol was dependent on cotransfection with the $G\alpha_{15,16}$ subunits (Figure 2C, right panel). Cells transfected with the G protein subunits alone (Figure 2C, left panel) produced a small response to isoproterenol, presumably due to some endogenous β-adrenergic receptors on their surface, but odorants such as heptanal (7-al) and octanal (8-al) had no effect (data not shown).

In a recent study (Zhao et al., 1998), the viral introduction of the full-length rat I7 receptor into olfactory neurons in vivo resulted in an increased electroolfactogram to octanal. As a second test example, we generated the Rho/$M4_{NC}$-ratI7 TM II–VII chimeric construct and coexpressed it with $G\alpha_{15,16}$ in HEK-293 cells. In agreement with the in vivo work, we observed in this experiment a Ca^{2+} transient in the transfected cells in response to 10 μM octanal (Figure 2D) but not to a prior application of 10 μM heptanal, a shorter aldehyde, although a response was seen to 30 μM (data not shown). The response to octanal required the presence of $G\alpha_{15,16}$ (Figure 2D, right panel). As with the β_2-adrenergic receptor, desensitization often occurred after a positive response. For example, in Figure 2D, little or no effect was observed upon a second application of octanal, even at 30 μM. A similar response profile was obtained with a construct in which the rhodopsin N terminus was fused to the full-length rat I7 coding region (Figure 2E). It responded to octanal even at 1 μM (data not shown). Again, the ligand specificity was not absolute, in that a small response was also observed to 30 μM heptanal, similar to the in vivo finding of Zhao et al. (1998). Sometimes the delay between the start of odorant application and the beginning of Ca^{2+} rise could be more than 30 s, such as for the first response to octanal in Figure 2E. The reason for this relatively long delay is unknown, but it could have arisen from a nonlinear, thresholding mechanism. Additional experiments in which successive applications of two odorants were separated by periods as long as 5 min, however, removed any possible confusion with respect to which odorant triggered a given response. Thus, for example, it was verified that the first response in Figure 2E was indeed to octanal and not heptanal.

The above results validate the HEK-293 cell expression system for identifying unknown odorants for the cloned receptor sequences and that ligand recognition can be largely imparted by TM II–VII of an olfactory receptor.

Identification of Cognate Ligand–Receptor Pairs for the Cloned Receptor Library

Eighty plasmid clones arrayed in microtiter plates were pooled into ten groups of eight constructs each and cotransfected with $G\alpha_{15,16}$ into HEK-293 cells. After loading with FURA-2, the transfected cells were screened sequentially against each of 26 odorants (Table 1), all at 10 μM, for an induced Ca^{2+} response as described previously. Three of the pools, I-3, I-6, and I-7, produced transient increases in Ca^{2+} in response to the application

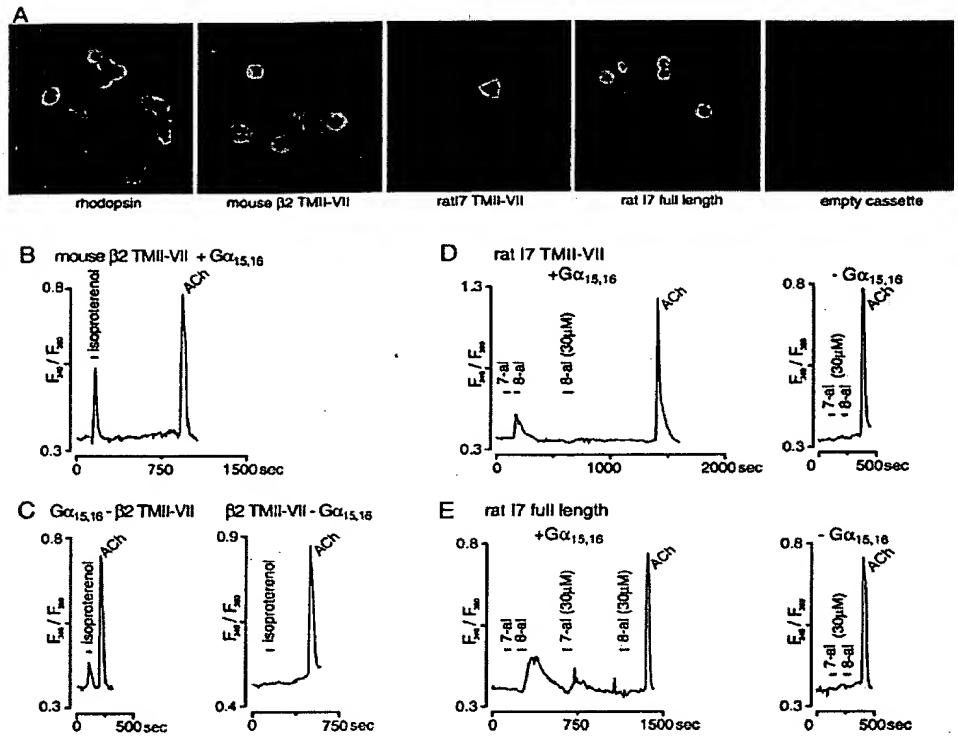

Figure 2. Coexpression of β_2-Adrenergic Receptor or Rat I7 Olfactory Receptor with $G\alpha_{15,16}$ Subunits Couples Receptor Activation to Intracellular Ca^{2+} Release

(A) Confocal images of HEK-293 cells transfected with rhodopsin and several rhodopsin-tagged receptor constructs. Immunostaining with the B6-30 monoclonal antibody against the rhodopsin tag reveals receptor expression near the plasma membrane.

(B–E) Ligand-induced intracellular Ca^{2+} increases in FURA-2-loaded HEK-293 cells cotransfected with the $G\alpha_{15,16}$ subunits and the pCMV-rho/$M4_{NC}$ mouse β_2 TM II–VII chimera (B), $G\alpha_{15,16}$ alone (C, left panel), mouse β2 TM II–VII chimera alone (C, right panel), $G\alpha_{15,16}$ subunits and pCMV-rho/$M4_{NC}$ rat I7 TM II–VII chimera (D, left panel), and $G\alpha_{15,16}$ subunits and the pCIS-rho rat I7 full-length coding region (E, left panel). The concentration used for all substances was 10 μM unless otherwise stated. The time and duration of the application of each substance is indicated by a horizontal bar. The Ca^{2+} signals were averaged from all responsive cells within the camera field (15 cells/total 96 cells in [B], 4/64 in [D], and 5/89 in [E]). The I7 TM II–VII chimeric and the full-length I7 receptors in the absence of $G\alpha_{15,16}$ did not induce a Ca^{2+} signal upon odorant challenge (D and E, right panels). With $G\alpha_{15,16}$ alone, cells (17/63) responded to 10 μM isoproterenol because of endogenous β-adrenergic receptors (C, left panel). Similar results were obtained in at least two additional, independent transfection experiments.

of (−) carvone, (−) citronellal, and (+) limonene, respectively (Figure 3). The lack of response of the I-3 pool to (+) carvone could reflect desensitization resulting from the positive response to (−) carvone occurring immediately before or, alternatively, a stereo specificity in ligand recognition (see later). This desensitization could also have obscured the response to subsequent odorant applications; nonetheless, a second response to (−) carvone could still be elicited. The absence of response to (+) citronellal for the I-6 pool apparently results from a genuine stereo specificity in ligand recognition, because there was no prior positive response that would lead to desensitization (also see below). The lack of responses to the subsequent odorants was confirmed by additional experiments with the same set of odorants, but where (−) citronellal was applied last. Finally, (−) limonene was not tried in the I-7 pool experiment because it was not among the 26 odorants initially tested (see Table 1).

Next, the eight clones in each of the I-3, I-6, and I-7 pools were tested individually for the respective odorants identified above. Three responsive chimeras were isolated in this manner, designated I-D3 (carvone), I-C6 (citronellal), and I-G7 (limonene). Further experiments indicated that the I-D3 receptor was responsive to both

Table 1. Odorants for Receptor Screening Experiments

1. Hedione	14. Pyrazine
2. (−)Carvone	15. 2-Methoxypyrazine
3. (+)Carvone	16. Isovaleric acid
4. (+)Citronellal	17. Isobutyric acid
5. (−)Citronellal	18. Tri-ethylamine
6. 2-Methyl-4-propyl-1, 3-oxalthiane	19. Citralva
	20. (+)Limonene
7. Methylsalicylate	21. 6-Aldehyde
8. Pyrrolidine	22. 7-Aldehyde
9. Quinoleine	23. 8-Aldehyde
10. Lyral	24. 9-Aldehyde
11. Cyclohexanone	25. 10-Aldehyde
12. Acetophenone	26. 11-Aldehyde
13. 2-Methoxy-3-Methyl-pyrazine	

Odorants 1–26 were applied in the indicated order for the initial screening experiments as shown in Figure 3.

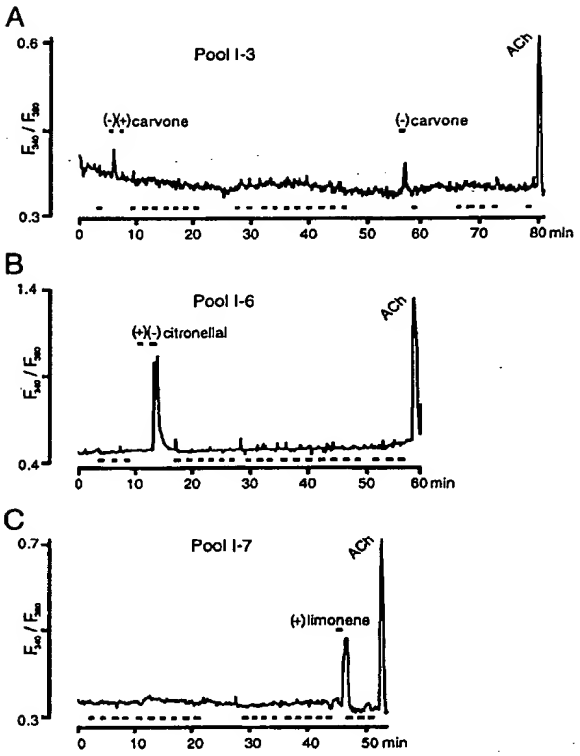

Figure 3. Screening of Pooled Chimeric Receptors with Odorants

HEK-293 cells cotransfected with DNA pools of eight receptor clones each and $G\alpha_{15,16}$ were tested after loading with FURA-2. The time and duration of the bath application of each odorant (10 μM) is indicated by a horizontal bar. The Ca^{2+} signals were averaged from all responsive cells within the same camera field (6 cells/total 44 cells in [A], 9/49 in [B], and 16/43 in [C]). The 26 odorants were applied in the order indicated in Table 1, except for an interposed, second application of (−) and (+) carvone, as well as pyridoxine instead of 9- and 10-aldehydes in (A). The odorants 9- and 10-aldehydes were missing in (B), and 6-, 9-, and 10-aldehydes were missing in (C). The responses of receptor pool I-3 to (−) carvone (A), receptor pool I-6 to (−) citronellal (B), and receptor pool I-7 to (+) limonene (C) were confirmed in at least two additional, independent transfection experiments.

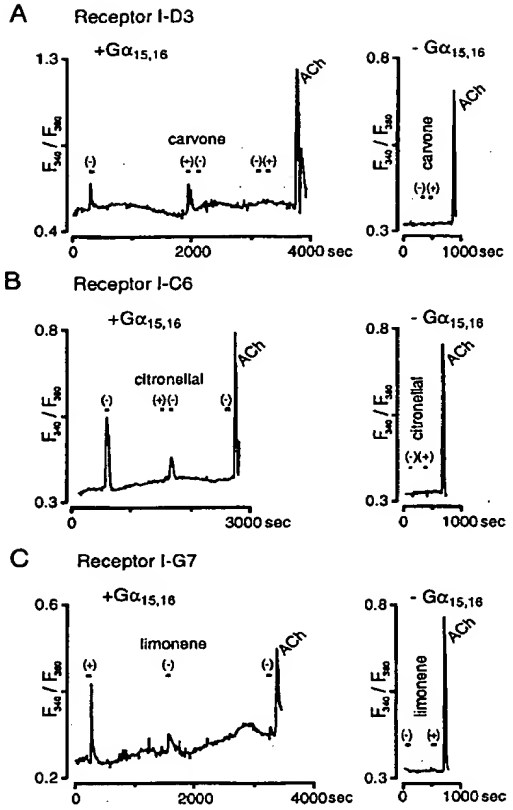

Figure 4. Identification of Single Chimeric Receptors Responding to Carvone, Citronellal, and Limonene

Same experimental procedure as in Figure 3, except only the I-C3 chimeric receptor from pool I-3 (A), the I-C6 chimeric receptor from pool I-6 (B), and the I-G7 chimeric receptor from pool I-7 (C) were cotransfected with $G\alpha_{15,16}$ into HEK-293 cells, and only the relevant odorants were applied. All odorants were bath applied at 10 μM. The response of the cells to carvone, (−) citronellal, and limonene, respectively, were confirmed. The Ca^{2+} signals were averaged from all responsive cells within the camera field (13 cells/total 58 cells in [A], 7/85 in [B], and 9/69 in [C]). The right panels show that odorant stimulation of ID3, IC6, and IG7 receptors in the absence of $G\alpha_{15,16}$ could not generate a Ca^{2+} signal. Similar results were obtained in at least two additional, independent transfection experiments.

(+) and (−) carvone (Figure 4A, left panel). On the other hand, the I-C6 receptor appeared to be selective for the (−) stereoisomer of citronellal (Figure 4B, left panel). Finally, the I-G7 receptor responded to both (+) and (−) limonene at the same concentration of 10 μM (Figure 4C), though perhaps not as well to the (−) isomer. For each of the three isolates, control experiments indicated that the specific responses required the presence of $G\alpha_{15,16}$ (Figure 4).

To confirm that the above responses were indeed representative of the corresponding full-length proteins rather than just the TM II–VII sequences in the chimeric constructs, we used the I-C6 receptor as a test case by isolating a genomic clone of its entire coding sequence and fusing it to the rhodopsin tag. The full-length I-C6 receptor retained the same stereo selectivity as the TM II–VII construct by preferring the (−) isomer of citronellal; it also showed high sensitivity, responding to this chemical even at 1 μM (Figure 5A). The stereo specificity was not absolute, however, in that (+) citronellal was also able to elicit a response when applied at 30 μM (Figure 5C, left panel) and 100 μM (Figure 5B). By comparison, carvone and limonene elicited no responses from this receptor, even at 100 μM (Figure 5B). Several structurally related compounds besides (−) and (+) citronellal were also tested (corresponding to the first five odorants applied in Figure 5C, left panel), all at 30 μM. Among these, only 30 μM (−) citronellyl bromide elicited a small response. This compound differs from (−) citronellal by the substitution of a bromine for the oxygen atom in the aldehyde functional group. The lack of response to (−) citronellal in Figure 5C (left panel) was presumably due to desensitization resulting from the positive response

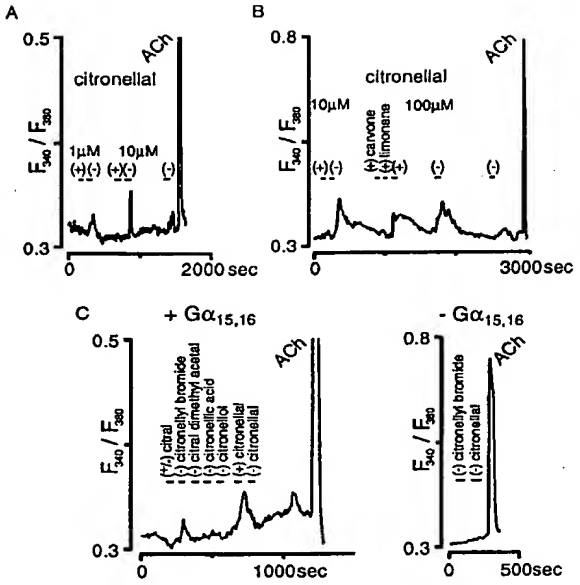

Figure 5. Concentration Range and Specificity of the Mouse Full-Length I-C6 Receptor

In HEK-293 cells cotransfected with $G\alpha_{15,16}$ and the rhodopsin-tagged I-C6 full-length coding region, a Ca^{2+} rise could be induced by (−) citronellal at 1 μM, 10 μM, and 100 μM (A and B), (+) citronellal at 30 μM (C, left panel) and 100 μM (B), and (−) citronellyl bromide at 30 μM (C, left panel). The Ca^{2+} signals were averaged from all responsive cells within camera field (7 cells/total 99 cells in [A], 7/92 in [B], and 7/99 in [C, left panel]). The origin of the Ca^{2+} rise before the ACh application in (C, left panel) is unknown. Without $G\alpha_{15,16}$, odorant responses were not observed (C, right panel). A horizontal bar indicates the time and duration of each bath application of odorant. Concentrations of odorants were 1 μM for the first two applications and 10 μM thereafter in (A), 10 μM for first two applications and 100 μM thereafter in (B), and 30 μM throughout in (C). The amplitude of the Ca^{2+} transient induced by 10 μM acetylcholine was 0.70 in (A) and 0.68 in (C, left panel). Similar results were obtained in at least two additional, independent transfection experiments.

to 30 μM (+) citronellal immediately before. Finally, in control experiments lacking $G\alpha_{15,16}$, no response was observed to either (−) citronellal or (−) citronellyl bromide (Figure 5C). Although these experiments do not permit a quantitative determination of ligand affinities, they provide a qualitative rank order of potency for binding and activating the I-C6 receptor: (−) citronellal > (+) citronellal; citronellyl bromide > 28 other odorants.

An Amino Acid Residue in TM V of the I7 Receptor Affects Odorant Specificity

In the course of experiments to establish the functional expression of mouse olfactory receptors, we also generated the Rho/$M4_{NC}$-mouseI7 TM II–VII chimeric receptor and examined its responsiveness to several n-aliphatic aldehydes and alcohols. To our surprise, at 10 μM concentrations of these odorants, the mouse receptor responded only to heptanal (Figure 6A, left panel; note that the background noise in this trace is relatively high and the fast spikes in the trace are not correlated with odorant applications). As described previously, the rat

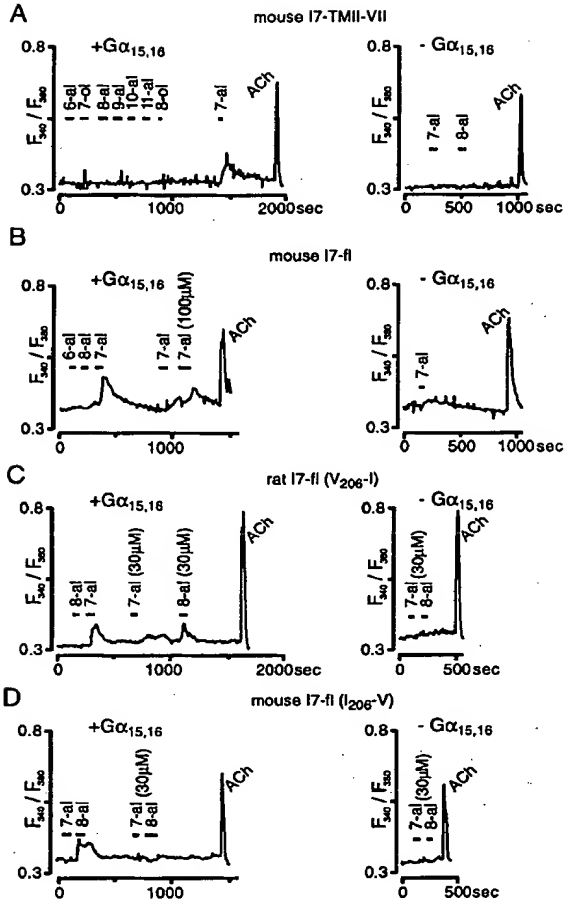

Figure 6. Amino Acid Position 206 in the I7 Receptor Is Important for Preference toward 7- or 8-Aldehyde

In HEK-293 cells cotransfected with $G\alpha_{15,16}$ and the mouse I7 TM II–VII chimeric receptor (A), the wild-type mouse I7 full-length coding region (B), and the mutated (V_{206}-I) rat I7 full-length coding region (C), a Ca^{2+} signal could be triggered by 10 μM 7-aldehyde. The full-length rat I7 mutant receptor was also responsive to 8-aldehyde at a higher concentration (30 μM) (C). The mouse I7 full-length receptor with the reciprocal mutation (I_{206}-V), on the other hand, responded to 10 μM 8-aldehyde but not 10 μM 7-aldehyde (D). The concentrations for all odorants were 10 μM unless otherwise indicated. A horizontal bar indicates the time and duration of each bath application of an odorant. The Ca^{2+} signals were averaged from 6 responding cells out of 86 in (A), 16/99 in (B), 8/89 in (C), and 4/66 in (D). The delays in the responses to the second and third applications of 7-aldehyde in (B) were unusually long. No Ca^{2+} signal induced by odorants was observed in the absence of $G\alpha_{15,16}$ (right panels in A–D). Similar results were obtained in at least two additional, independent transfection experiments.

I7 chimeric receptor responded better to octanal than to heptanal in identical experiments (Figure 2D, left panel). This difference in odorant selectivity was retained by the full-length clones of the two receptors fused to the rhodopsin tag (Figures 2E and 6B, left panels). The rat and mouse I7 receptors differ in altogether 15 amino acid residues, three of which (K_{90}E in the first extracellular loop, V_{206}I in TM V, and F_{290}L in TM VII) reside between

TM domains II and VII. In light of the critical role of residues in TM V for ligand binding in the β_2-adrenergic receptor, we examined the role of residue 206 in differential ligand recognition. Reciprocal valine/isoleucine substitutions were made in the full-length rat and mouse I7 receptor sequences. These substitutions were able to switch the ligand preferences of the two receptors, namely making the rat I7 receptor preferentially recognize heptanal and the mouse receptor preferentially recognize octanal (Figures 6C and 6D, left panels). Interestingly, the nature of these changes, isoleucine versus valine and heptanal versus octanal, is consistent with compensatory alterations in the structures of ligand and receptor that preserve the complementarity between the two. These observations provide strong evidence for a direct role of residue 206 in the interaction between the I7 receptor and aliphatic aldehydes.

Discussion

Expression Cloning Approach

The few studies carried out previously on identifying cognate odorant–olfactory receptor pairs have generally focused on a single receptor and examined its responsiveness to a large number of odorants or odorant mixtures (see Introduction). We have taken a different approach by generating an olfactory receptor library and screening in parallel a number of cloned receptors against a panel of individual odorants. In this way, one is more likely to get around the problem of poor expression, inefficient folding, or weak coupling to second-messenger systems associated with certain receptors in a heterologous system. Moreover, by screening multiple receptors against multiple odorants, one greatly increases the probability of identifying responsive combinations of receptors and odorants. Finally, the apparent diversity of the receptor sequences should further enhance the scan of the odor space. In our experiments, we have screened 80 clones (not counting the I7 receptor) against 26 odorants. Because a given odorant should be recognized by at least one member of, say, a total of 1000 receptors, the chance of encountering an odorant that is a cognate ligand to 80 receptors should, on average, be 8% (80/1000), or 2 positives in a pool of 26 odorants. This number is not too far off from the number (3) we have identified experimentally. While interesting, these estimates are too crude and the sample size too small at present to derive biological inferences. Furthermore, there is a lack of knowledge regarding the range of odor space reflected in the test panels and the partitioning of odor space across the repertoire of receptor proteins.

The design of the olfactory receptor expression cassette used in these experiments was guided by the notion that the TM II–VII region of some known G protein-coupled receptors define the ligand-binding pocket (see Introduction). This principle appears to be applicable to the three olfactory receptors that we have specifically examined, namely the rat and mouse I7 receptors and the mouse I-C6 receptor. For example, the full-length and the TM II–VII chimeric rat I7 receptors both recognize octanal and prefer it to shorter and longer aldehydes. Likewise, the full-length and the TM II–VII chimeric mouse I-C6 receptors both recognize citronellal and, furthermore, prefer (−) citronellal to the (+) isomer. It is difficult to generalize from just three receptors to the rest of the family. However, more light can be shed on this question by rescreening the 80 clones we have studied against a larger panel of odorants and by expanding the screen to the 400 untested clones in the library. Finally, it is likely that the ongoing efforts in other laboratories to elucidate the genomic organization and DNA sequences of olfactory receptors will converge with the functional analysis described here to increase the number of examples where the ligand recognition properties of the chimeric and full-length receptors can be examined. It may turn out, for example, that sequences in TM I are required for ligand binding to some receptors. Moreover, the availability of high-throughput, Ca^{2+}-release-based screening protocols will provide the opportunity to greatly expand the matrix of known ligand–receptor interactions.

As described earlier, the receptor library that we have generated with a single pair of degenerate primers in TM II and TM VII encompasses a broad range of the olfactory receptor family. Preliminary sequencing studies of additional clones from the array (data not shown) are consistent with several hundred distinct sequences being represented in this library.

The addition of a rhodopsin N-terminal segment (the 5′-untranslated region and the first 20 amino acid residues) to the cloned receptors appears to facilitate the surface localization of the cloned receptors. While we have not tested this idea extensively, it appeared to be the case for the full-length I-C6 receptor, where the inclusion of the rhodopsin tag was necessary in order to observe a response to (−) citronellal (data not shown). Although a direct contribution by the rhodopsin tag to the odorant response still cannot be ruled out, its distance from the presumed ligand recognition domain, as well as the agreement in ligand specificity for the rat I7 receptor between in vivo experiments and our heterologous expression experiments, would tend to argue against this possibility. Immunocytochemical experiments with another full-length receptor, M4, have suggested that the surface localization of the receptor may indeed be improved by the rhodopsin tag (R. R. R., unpublished observations). It is unclear whether this facilitation results from a structural contribution by the rhodopsin tag to the protein translocation process or from a higher level of protein expression due to the rhodopsin translation initiation site. Recently, a construct was made in which a 5-$HT1_c$ receptor N-terminal sequence was appended to the N terminus of fish olfactory receptors (Wellerdieck et al., 1997) in an effort to functionally express vertebrate odorant receptors.

In our experiments, a single application of a cognate odorant to an olfactory receptor in HEK-293 cells very frequently led to a complete and long-lasting desensitization to further ligand application. In contrast, the response to an application of acetylcholine at the end of an experiment was invariably robust. Furthermore, successive applications of acetylcholine continued to produce a response. Thus, the olfactory receptor desensitization perhaps resides at the receptor or G protein levels. Because of this desensitization and the signal

averaging over multiple cells, positive responses subsequent to the first should be interpreted qualitatively but not quantitatively.

Ligand Selectivity

The I-C6 receptor appears to prefer (−) citronellal to (+) citronellal. On the other hand, the I-D3 receptor is activated by both stereoisomers of carvone. Psychophysical studies have demonstrated that humans find the two isomers of citronellal to possess similar odorant qualities, but can easily discriminate between (+) carvone (caraway) and (−) carvone (spearmint). One explanation for these discrepancies is that mouse and human differ in their odorant-discriminating abilities. As shown by our experiments (and see below), even the mouse and rat I7 receptor orthologs can differ in their odorant specificity by virtue of a single conservative residue change. For carvone, it is also possible that, in addition to I-D3, there is another olfactory receptor that shows stereospecificity to carvone. Signals from both receptors can thus code for general and stereo recognition of carvone, respectively. In the case of citronellal, the I-C6 receptor may be of minor importance for the recognition of citronellal in vivo, with this function being delegated to a higher-affinity receptor incapable of stereorecognition.

The above considerations raise the point alluded to earlier that each receptor may respond to a broad spectrum of odorants, with possibly considerable overlap between receptors. Because we have used only a small set of odorants for our screening, we may have missed the primary odorants for the I-D3, I-C6, and I-G7 receptors. Although these receptors can detect carvone, citronellal, and limonene at low micromolar concentrations, the detection threshold for some odorants in mammals can be as low as a few parts per billion. Multiple factors may explain the higher sensitivity observed in vivo, including the presence of odorant carrier proteins in the nasal mucus (Pevsner et al., 1988), the specialized structure of the olfactory receptor neuron, high densities of olfactory receptors on the cilia, a high-gain coupling of the native olfactory transduction components, and an improved signal-to-noise ratio afforded by the convergence of many receptor neurons expressing the same receptor protein to a single glomerulus in the olfactory bulb (Vassar et al., 1994). On the other hand, because the overall odor space is so large, there may indeed be ligands that display higher affinities than carvone, citronellal, and limonene for the I-D3, I-C6, and I-G7 receptors. The ability to disrupt the gene coding for an olfactory receptor of known ligand specificity in the mouse genome should permit direct demonstration of the importance of an identified receptor in the detection of particular odorants. Similarly, the functional assay described here may be used for identifying the cognate odorants for those olfactory receptors that have been the targets of genetic manipulations.

Differential Ligand Recognition by Orthologous Receptors

The I7 receptors from mouse and rat display 94% amino acid identity and represent the orthologous gene in the two species. Despite this conservation, we have found that the two orthologous receptors do not exactly correspond in their odorant response, in that the rat I7 receptor prefers octanal to heptanal, and the reverse for the mouse I7 receptor, a difference accountable by a single-residue change in position 206. This change in ligand specificity resulting from a minimal change in the primary structure of an olfactory receptor may be an illustration of the positive Darwinian selection for nucleotide changes described for the olfactory receptors in catfish (Ngai et al., 1993).

Experimental Procedures

Chimeric Receptor Cassette for Eukaryotic Expression

The chimeric receptor expression vector was assembled from a modified pBK-CMV plasmid (Stratagene) that had the *lacZ* sequences between nucleotides 1098 and 1300 deleted. A PCR fragment consisting of 45 nucleotides upstream of the bovine rhodopsin initiation codon and the first 60 nucleotides of the coding region (rhodopsin tag) was introduced between the BamHI and EcoRI sites. Restriction fragments corresponding to the first 57 amino acids (N terminus to TM II, EcoRI/PstI) and the last 22 amino acids (BspEI/XbaI) of the mouse M4 olfactory receptor (Qasba and Reed, 1998) were cloned into the rhodopsin tag vector. The resulting vector (pCMV-Rho/$M4_{NC}$) possesses unique PstI and BspEI sites at the beginning of TM II and the end of TM VII, respectively (see Figure 1).

PCR Amplification of Fragments for Receptor Coding Regions and Vector Construction

PCR amplification was performed with a mixture of *Taq* and *Pfu* polymerase (Stratagene) (0.5 U each), 0.2 mM dNTP, 1 μM of each primer, and either 100 ng mouse genomic DNA (β_2-adrenergic receptor), 10 ng plasmid template DNA, or 50–100 ng first strand cDNA template prepared from mouse (C57BL/6J) olfactory epithelium. The amplification protocol was 1 × 2 min at 94°C; 30×(55°C, 72°C, 94°C), 1 min each; 1×(55°C, 72°C), 10 min. The oligonucleotides used for PCR amplification of the various sequences are available from the authors.

Generation of Mouse Olfactory Receptor Transmembrane II–VII Library

A 0.7 kb PCR product was generated using the following degenerate oligonucleotides:

dk-71-GGGGTCCGGAG(A/G)(C/G)T(A/G)A(A/G/T)AT(A/G/P)A(A/G/P)(A/G/P)GG

dk-72-GGGGCTGCAGACACC(A/C/G/T)ATGTA(C/T)(C/T)T(A/C/G/T)TT(C/T)(C/T)T

P: dP-CE Phos-phoramidite (Glen Research). The amplification protocol was 1 × 2 min at 94°C; 34×(45°C, 72°C, 94°C), 1 min each; 1×(45°C, 72°C), 10 min.

The PCR product was digested with PstI and BspEI before size fractionation, purification, and ligation into the pCMV-Rho/$M4_{NC}$ vector (Figure 1A). The ligation products were transformed into *E. coli*, and 480 clones were placed in 96-well plates. PCR screening revealed that >95% of the clones carried inserts of the expected size. Pools of cells from a single column of the plates (8 wells) were grown in a 50 ml culture and plasmid DNA prepared.

Cloning of the Full-Length Mouse I7 and IC-6 Olfactory Receptors

The full-length coding region of olfactory receptors mI7 and I-C6 were obtained by screening a mouse (129 SV/J) genomic phage (λFIX-II) library (2 × 10^6 independent clones) using ^{32}P-labeled DNA fragments (TM II–VII) of the respective receptors under stringent conditions. DNA fragments encoding the full-length receptors were cloned into pBluescript and sequenced.

Culture and Transient Transfection of HEK-293 Cells

HEK-293 cells were grown in DMEM supplemented with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and

L-glutamine (2 mM) in 5% CO_2. Before transfection, the cells were seeded onto poly-L-lysine-coated 10.5×35×0.17 mm glass coverslips (Bellco) placed in the 60 mm culture dishes. Calcium phosphate–mediated transfections were performed in a 60 mm dish with 3–4 μg of receptor construct DNA, 1 μg of pCIS $G\alpha_{15}$ and $G\alpha_{16}$ expression vector, 2 μg of pBluescript carrier DNA, and 0.3 μg of pRSV-T antigen expression vector. After 5–7 hr incubation, the cells were washed once with PBS containing 0.5 mM EDTA and 10% DMSO and then with PBS before continuing growth in regular media for 40–50 hr (Levin et al., 1992).

Immunocytochemistry

Transfected HEK-293 cells were air dried and fixed in ice-cold methanol for 10 min. The fixed cells were blocked with 1.5% goat serum in PBS for 30 min and then incubated for 1 hr in PBS containing 0.03% goat serum and a 1:1000 dilution of the B6-30 mouse monoclonal antibody (Hargrave et al., 1986). An FITC-coupled, polyclonal anti-mouse antibody (Vector) was used to visualize the rhodopsin-tagged protein. Images of fluorescent cells were obtained on a Zeiss 510 confocal microscope with excitation at 488 nm.

Single-Cell Ca^{2+} Imaging

Cells were loaded with Ca^{2+}-sensitive fluorescent dye by bathing in serum-free DMEM containing 4 μM FURA-2 AM (Molecular Probes) for 1 hr at 37°C, then washed with a standard bath solution (130 mM NaCl, 2 mM $CaCl_2$, 5 mM KCl, 10 mM glucose, 10 mM Na HEPES [pH 7.4] at room temperature). For each experiment, a glass coverslip with FURA-2-loaded HEK-293 cells was introduced into an open-topped, longitudinal microperfusion chamber (300 μl bath volume) mounted on a Zeiss Axiovert 135 microscope equipped with a F Fluar 40×/1.30 oil-immersion lens. The cells were superfused with test solutions typically for 30–40 s (5 ml/application) and washed out with 5 ml of bath solution at the end of each application. Each test solution was freshly diluted and manually applied with a micropipette into the chamber. Because of this manual procedure, there could be several seconds delay in actual application from electronic tick marks used to define the beginning of application in each graph. At the same time, the solution flow might not be completely laminar. In most cases, the onset of Ca^{2+} rise in response to a specific solution occurred within 15 s of the beginning of solution application, though longer delays were sometimes observed (see Results). Acetylcholine was applied at the end of each experiment at 10 μM for 15–20 s. Ratiometric Ca^{2+} measurements were performed as previously described (Grynkiewicz et al., 1985), with modifications using the Zeiss/Attofluor-Ratiovision imaging system. At 5 s intervals, the cells were sequentially illuminated for less than 100 ms, first at 340 nm, and then at 380 nm. Fluorescence emission at 510 nm was monitored for each excitation wavelength via an intensified CCD camera. Averaged pixel intensities within 40–100 regions of interest, corresponding to 40–100 individual cells, were digitized and stored on a personal computer. Attofluor-Ratiovision software (Atto Instruments) was used to determine the Ca^{2+}-dependent fluorescence signal expressed as the F_{340}/F_{380} ratio. Signals from all responding cells or all cells (negative controls) were averaged and displayed as a function of time.

Odorants

The odorants were kindly provided by Dr. C. Margot (Firmenich S. A.) or purchased from other sources (Table 1) and stored under nitrogen. Stock solutions of the odorants were made up fresh each day in DMSO and diluted 1000-fold into the standard bath solution to give the indicated concentrations ca. 10 s before application in a given experiment.

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